ROCK-dependent mechanotransduction of macroscale forces drives fibrosis in degenerative spinal disease

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INTRODUCTION: Chronic repetitive forces on the spinal column promote the development of degenerative spinal disease (DSD); yet, the mechanism linking macroscale mechanical forces and tissue hypertrophy remains unknown. In degenerative spinal disease (DSD), mechanical stress causes fibrotic changes in ligament and bone, with resultant compression of neural elements [1, 2]. Global studies predict 84% of people will be affected by DSD [3], with the ultimate treatment often requires surgical decompression and fusion, with multimodal treatment costs reaching $13 billion annually in the United States alone [4]. Understanding how macroscale forces affect fibrotic changes in the spine is necessary to develop non-operative disease-modifying therapies for DSD. We report elevated Rho-associated kinase (ROCK) signaling and an increased density of smooth muscle actin alpha (SMAα) myofibroblasts in regions of fibrosis in human ligamentum flavum (LF) naturally exposed to high stress. With Atomic Force Microscopy (AFM) we localize myofibroblasts to regions of elevated stiffness and microstress within the LF. With a novel loading device, stress-dependent LF myofibroblast accumulation was observed in a ROCK-dependent manner. Finally, we demonstrate stress-driven transcriptional response in human LF, partially reduced by ROCK inhibition. These findings warrant detailed future investigation into ROCK inhibitors as a potential class of compounds to treat DSD in a non-operative manner.

METHODS: Ligamentum Flavum (LF) samples were collected from over 250 patients who received spinal surgeries at Massachusetts General Hospital (MGH). Samples were collected within 30 minutes of surgical removal and were transported in a sterile specimen cup on ice in fewer than 15 minutes. Samples were processed and stored differently for each experimental method, as detailed in the following sections. All samples were collected under IRB protocol 2017P000635. While different samples from the same patients were used for varying simultaneous experiments, no samples were reused. A millimeter-scale custom transparent, numbered grid was designed and printed on transparency film, producing a square grid sized 2 cm x 2 cm (**Figure 1**). Using a cyanoacrylate adhesive, one grid was attached to the bottom of each microscope slide. The desired ligament samples were frozen promptly in cryomolds with OCT, cryosectioned at a 20µm thickness, and placed on the microscope slide's surface to be centered on the grid underneath the slide. Using the Evos stitching microscope, the whole microscope slide was imaged to record the exact placement of the ligament in relation to the grid. To measure the modulus of samples we used a commercial atomic force microscope (AFM)(MFP-3D, Asylum Research, Santa Barbara, CA). We used glass probe tips with varying diameters of 20–50µm attached to tipless cantilevers with nominal spring constant k ~ 10 N/m (Budget Sensors, Sofia, Bulgaria). Spring constants were directly measured for all tips using the thermal calibration method. The glass probe tips were attached to the cantilever by the lift-off process. Locations on the slide were imaged, then indented in a systematic manner mapping the full spatial extent of a given slice/sample.

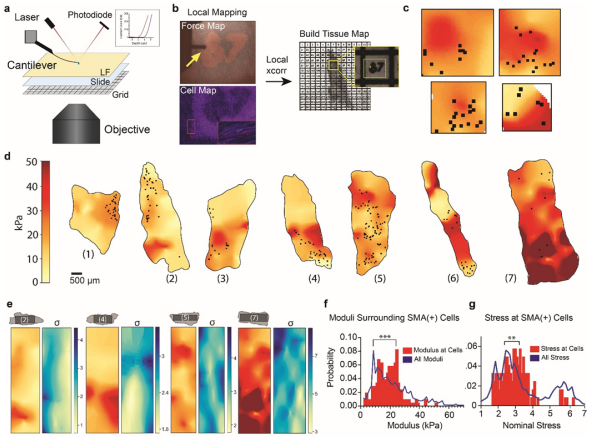
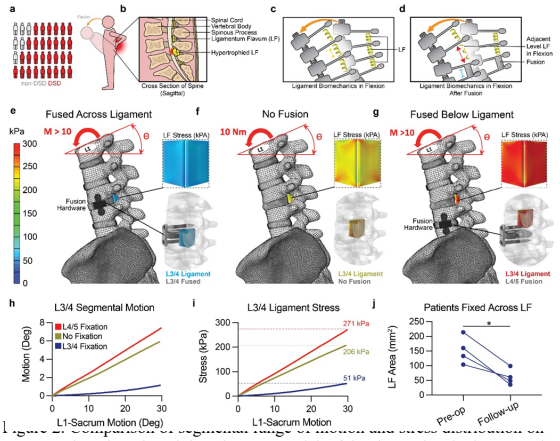


Figure 1. Myofibroblasts localize within LF to regions of higher stiffness and higher simulated stress.

After completing AFM, the ligament was gently fixed and immunofluorescent staining was performed for SMAα (as detailed above). Upon completion of the IF staining, the slide was re-imaged on the Evos stitching microscope to ensure the tissue slice did not move in relation to the grid and then a blinded investigator inspected the ligament slice and recorded the exact locations of each SMAα cell. A second independent investigator designed and utilized a custom MATLAB code to create modulus maps and to then superimpose the locations of SMAα cells on the fabricated maps. To understand the changes in load distrbution on LF in fixation construct we used utilized a validated finite element model of the L1 to pelvis spine [5]. The model incorporated 3D geometries of vertebrae, intervertebral discs, facet joints, and spinal ligaments, including the ligamentum flavum. The ligamentum flavum was modeled using a fiber-reinforced hyperelastic material law, with collagen fiber orientation captured. Material parameters were calibrated to match experimental data on ligamentum flavum tensile stiffness and mechanical properties. Three cases were analyzed: intact, L4/L5 fusion, and L3/L4 fusion. For the fusion cases, virtual models of lateral PEEK interbody cages and titanium plates/screws were implanted following simulated lateral discectomy. Surfaces were tied to simulate bone-implant fixation. The intact model underwent a 400N follower preload plus flexion, extension, lateral bending, and axial rotation moments up to 10Nm. The fusion cases used a hybrid loading protocol with 400N follower load and moments adjusted to match intact range of motion. Following simulation, intact and fused segmental range of motion was computed and compared. The ligamentum flavum was analyzed to determine macroscale principal stresses and strains. Ligamentum flavum stress distributions were compared between intact, fused, and adjacent unfused levels to assess biomechanical changes. This validated computational approach enables detailed quantification of how fusion alters load transfer to the ligamentum flavum.



LF at L3/4 using the Finite element model of lumbar spine in intact and instrumented with lateral fixation system at L3/4 and L4/5 levels.

RESULTS: Fusion of vertebrae adjacent to the LF caused increased peak segmental motion and adjacent LF stress compared to the unfused spine by 31% and 25%, respectively (Figure 2). Increased LF stress corresponded to increased segmental motion, which was necessary to compensate for the adjacent immobilized (fused) spinal level to maintain the same global degree of flexion. Fusion across LF caused decreased peak segmental motion and stress on the immobilized LF compared to the normal spine by 81% and 75%, respectively (Figure 2). Retrospective chart review revealed that immobilization of levels across the LF.

DISCUSSION: we demonstrate stress-driven transcriptional response in human LF, partially reduced by ROCK inhibition. These findings warrant detailed future investigation into ROCK inhibitors as a potential class of compounds to treat DSD in a non-operative manner.

SIGNIFICANCE: Clinical stress risers and in vivo cyclic stress drive rescuable, ROCK-dependent myofibroblast accumulation in spinal ligament contributing to degenerative spinal disease.

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